Comm.
Dr. Jacobson
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CHRONIC PULMONARY DISEASES

THE COUNCIL FOR TOBACCO RESEARCH - U.S.A.

110 East 59th Street
New York, N. Y. 10022



Application For Research Grant

Date: January 30, 1973

#764B
#764R2-7/1/72-6/30/73
#764R1-7/1/72-6/30/72
#764S - 9/70
#764 - 7/1/70-6/30/71

1. Name of Investigator(s): (include Title and Degrees)

Charles G. Cochrane, M. D. Member
Peter M. Henson, Ph. D., Associate Member
Stephen W. Russell, D. V. M., Ph. D. - Research Fellow
Thomas C. Kravis, M. D. - Research Fellow

2. Institution & Address:

Department of Experimental Pathology
Scripps Clinic and Research Foundation
476 Prospect Street
La Jolla, California 92037

3. Short Title of Project: The Mediation of Inflammatory Injury of Tissue

4. Proposed Starting Date: July 1, 1973

5. Anticipated Duration of this Specific Study: 3 years

6. Brief Description of Objectives or Specific Aims: The proposed studies will examine the mechanisms by which inflammatory injury of tissue occurs. Both humoral and cellular factors will be examined:

1. To study the mechanism of activation of the kinin forming, intrinsic clotting and fibrinolytic systems. In the previous three years of support by the CTR, the initial components of each of these systems has been isolated in highly purified form in the native (precursor) state from human and rabbit plasma. These components have been radiolabelled and antibodies have been prepared against each. We are now in a position to examine in detail the participation of these systems in inflammatory injury of tissue.

2. Studies of inflammatory injury mediated by leukocytes and other cells. The participation of cellular reactants in inflammatory injury will be studied. The activation, mechanism of release and modulation of these phenomena will be studied.

7. Give a Brief Statement of your Working Hypothesis: Inflammatory injury, induced by a wide variety of stimuli, is effected by a series of humoral and cellular mediators. While in many instances the causative inciting agents remain unknown, the mediation systems of the injury are common to them all and subject to analysis. Through analysis and understanding of these systems, inhibitors of the sequential injurious events can be sought and disease prevented.

8. Details of Experimental Design and Procedures: (Attach Separate Pages)

Scientists have undertaken two general approaches to the problem of inflammatory injury of tissue, the first involving a search for inciting agents that initiate the disease such as microorganisms, chemical agents, specific antibodies, etc., and the second, involving the analysis of the host factors, brought into the arena by the inciting agent, that are responsible for mediating the structural injury of tissue. These host factors include the humoral and cellular mediation systems. In many inflammatory diseases currently under investigation the inciting agents remain an enigma. Such is the case in rheumatoid arthritis, certain forms of pulmonary fibrosis, thyroiditis, multiple sclerosis, polyarteritis nodosa, to name only a few diseases. Single or combined inciting agents have not been detected consistently despite a vast effort. The reasons for this are complex and possibly specific to each disease. (continued on page 5)

9. Physical Facilities Available (Where Other than Administering Organization Indicate Geographical Location)

Approximately 2000 sq. ft. of laboratory space is available. The laboratory is equipped with refrigerated centrifuges, electrophoretic and chromatographic equipment, five fraction collectors, a walk-in cold room, deep freezes (-20° and -70°), complete fluorescent equipment and a preparative ultracentrifuge. Histologic and electron microscopic analyses are carried out in facilities available and frequently used by the applicants. An analytical ultracentrifuge and amino acid analyzer are also used by the applicants within the department. Complete isotope labeling, hot lab and monitoring facilities are in current use. Two Schultz-Dale bath apparatuses are used for assys of biologically active materials.

10. Additional Requirements:

None

11. Biographical sketches of all principal and professional personnel (append)

appended

12. List of publications: (Five most recent as pertinent) (append)

appended

13. Budget: (1st year)

| A. Salaries (Personnel by names) | % time | Amount |
|---|---|---|
| Professional | | |
| Charles G. Cochrane | 20 | -0- |
| Peter M. Henson | 20 | -0- |
| Stephen W. Russell | 50 | 8,000 |
| Technical | | |
| Lynette Buettner | 100 | 7,500 |
| Dishwasher | 30 | 2,000 |
| Animal Caretaker | 30 | 2,200 |
| | Sub-Total | 19,700 |
| B. Consumable Supplies (list by categories) | | |
| Chemicals, proteins | | 5,200 |
| Glassware, plasticware | | 5,200 |
| Animals, feed and bedding | | 1,500 |
| | Sub-Total | 11,900 |
| C. Other Expenses (itemize) | | |
| Travel, 1 fellow to East Coast meeting | | 450 |
| Reprints | | 900 |
| | Sub-Total | 1,350 |
| D. Permanent Equipment (itemize) | | |
| E. Overhead (15% of A+B+C) | | 4,942 |
| | Total | 37,892 |

Estimated Future Requirements:

| | Salaries | Consumable Suppl. | Other Expenses | Permanent Equip. | Overhead | Total |
|---|---|---|---|---|---|---|
| Year 2 | 20,100 | 11,900 | 1,350 | | 5,002 | 38,352 |
| Year 3 | 20,600 | 11,900 | 1,350 | | 5,077 | 38,927 |

Signature Charles G. Cochrane
Director of Project (714) 459-2390

Signature Edmund L. Kenney
Business Officer of the Institution (714) 459-2390

## Other Sources of Financial Support

List financial support for research from all sources,
including own institution, for this and/or related research projects

Current

| Title of Project | Source | Amount | Duration |
|---|---|---|---|
| Immunologic Studies | United States Public Health Service | 150,000 | 1973-74 |
| The Mediation of Inflammatory Injury of Tissue | Council for Tobacco Research | 31,269 | 1972-73 |

(This grant supports entire costs of research of seven full time investigators - including the three applicants, eight technicians, two animal caretakers, a glassware washer and one secretary)

4.

Pending None

8. Details of Experimental Design and Procedures: (Continued) - 2

While efforts must continue to gain an understanding of the inciting agents, the difficulties encountered to date underscore the importance and urgency of investigating the mediation systems of inflammatory injury. In the mediation systems one finds many features common to various inflammatory diseases, such as the participation of complement components, leukocytes and effector molecules. Knowledge of one system, may well apply to the mediation of a second. And through analytic studies, the members of the mediating pathways become characterized and thereby vulnerable to specific pharmacologic attack. Thus the pharmacologic inhibition of the third component of complement, the prevention of release of lysosomal constituents of neutrophils to the surrounding milieu or the inhibition of released leukocytic protease may signal a new era of specific therapy.

This laboratory has been engaged for a number of years in the analysis of several of the mediating sequences. A portion of this effort has been supported by the Council for Tobacco Research. We propose now to further these investigations and in particular, to apply a number of findings from in vitro studies to models of disease in vivo. It is our contention that such studies are essential to the understanding of inflammatory injury of the lung brought about by any inciting agent. And such studies will hopefully lead to rational therapy of inflammatory disease of the respiratory system.

1. Studies of the fibrinolytic, intrinsic clotting and kinin forming systems in inflammatory injury.

In our previous studies, the initial members of these systems in rabbit and human plasma have been isolated, purified and characterized. Antibodies have been produced to each. A summary of progress has been appended (Appendix A) and the sequence of action tabulated (Table I and Fig. 1). The activation of the initial component, Hageman factor, follows binding to various membranes such as vascular basement membranes or collagen bundles, or is caused by enzymatic action of kallikrein, plasmin and clotting factor XI (listed in order of potency). The marked enzymatic activation in fluid phase by kallikrein was a surprising finding and may well represent the major mechanism by which Hageman factor is activated in vivo (See Appendix A).

8. Details of Experimental Design and Procedures: (Continued) - 3

a. Activation of Hageman factor on membrane surfaces.

In order to gain a better understanding of the surface activation of Hageman factor on vascular structures a series of experiments will be conducted to detect binding and activation of Hageman factor in vivo along vascular and extravascular membranes. Preliminary data from this and other laboratories have revealed that Hageman factor does bind and is activated by vascular basement membranes and collagen. We now have produced strong monospecific antibody to rabbit and human Hageman factor that have been conjugated with fluorescein. This will be employed to detect the exact position in sections of lung where Hageman factor binds. In addition, the binding of Hageman factor will be examined in acute inflammatory pneumonitis produced by antibody directed to rabbit pulmonary membranes. Hageman factor bound indiseased human lung will also be sought in experiments to be performed with Dr. Averill Liebow of the University of California, San Diego. Early studies will be directed toward acute and chronic inflammatory processes.

Investigation will also be conducted into the structure of extracellular membranes responsible for the binding and activation of Hageman factor. Extracellular membranes will be obtained from several sources: pulmonary alveolar membranes, collagen, vascular basement membrane (glomerular), basement membrane glycoprotein secreted by a parietal yolk sac carcinoma that has been adapted to tissue culture by Dr. Lewis Johnson (who has kindly provided the purified glycoprotein). In addition, a series of synthetic peptides bearing triplet amino acid residues common to groups of amino acid residues in collagen have been obtained from Dr. Darwin Prokop of Rutgers University. These peptides and several analogues will be employed in blocking experiments (they do not activate Hageman factor) designed to inhibit interaction of Hageman factor and the various membrane structures.

These extracellular membranes will be treated with various agents in order to solubilize peptide moieties. The soluble products will then be tested for binding and activation of Hageman factor or for inhibition of this process by isolated vascular basement membrane (prepared in highly purified form from rabbit and human glomeruli in this laboratory). Among the agents to be used for solubilization will be purified specific collagenase, trypsin and cyanogen bromide. Considerable experience in isolating soluble fragments of extracellular membranes has been gained in this department in the past several years.

These studies should provide considerable information on the position in normal and diseased pulmonary tissue where Hageman factor may be activated, and the structure of the membrane responsible for binding and activation. The information will provide a foundation upon which a greater understanding of the participation of the intrinsic clotting, fibrinolytic and kinin forming systems participate in pulmonary disease. The fact cannot be stated too strongly that essentially no firm information exists today regarding to the importance of these systems in inflammatory disease despite the immense potentiality of products of these systems to induce injury.

8. Details of Experimental Design and Procedures: (Continued) - 4

b. The effect of inhibitors of the Hageman factor activated systems on the development of inflammatory disease. Employing the model of pulmonary and glomerular inflammation induced by heterologous antibody to the respective membranes, various inhibitors of the kinin forming, intrinsic clotting and fibrinolytic systems will be tested for their ability to inhibit development of disease. Assays of inflammatory injury will include histologic damage to cells and membranes, deposition of fibrin, accumulation of leukocytes and intravenously injected colloidal carbon along vascular membranes indicating increased vascular permeability. In the case of glomerular injury, proteinuria will be measured quantitatively. In each case, the antibody will be labelled with $^{125}I$ and its binding in the tissues measured (using $^{131}I$ normal globulin as a paired-label control) in order to control the quantity of inciting agent participating in the reaction.

Inhibition will be conducted by mediation systems. It is known that both pulmonary and glomerular injury have two mediation pathways, one requiring complement and neutrophils, and another producing injury in the abscence of these. We have found previously that the neutrophil-complement independent injury of glomeruli is qualitatively different from the injury resulting from the participation of these mediators in experimental glomerulo-nephritis. Thus inhibition experiments will be conducted in both the abscence and presence of neutrophils. Pulmonary and glomerular inflammation will be produced by injection of $^{125}I$ antibody so that the quantity of bound antibody can be assessed. Inhibitors will then be tested in various groups of rabbits: the fibrinolytic pathway will be blocked by injection of epsilon aminocaproic acid or Mercke compound 576 which prevent activation of plasmin. Fibrin will form along glomerular and pulmonary vascular membranes as we have observed by both light and electron microscopy. The amount of fibrin should be much greater in treated animals. However with the inhibition of plasmin generation, a corresponding decrease in the rate and quantity of formation of fibrin split products will be observed. The presence of split products will be monitored by injecting $^{131}I$ rabbit fibrinogen with detection of labelled peptides in the urine and plasma. Split products in plasma will also be measured by their capacity to bind to and precipitate polyanions and plasmin generation at the site of injury assessed by the micro-fibrinolytic assay with frozen sections of tissue. The assay of plasmin activity is not quantitative, but will allow us to determine if fibrinolytic (plasmin) activity is present.

These studies will provide evidence on the importance of plasmin formation in the development of injury in both normal and neutrophil depleted animals, and indirectly of the role of fibrin split products. Control studies will be conducted on possible alternative effects of EACA and Mercke compound #576: inhibition of activation of the complement system, immune adherence of neutrophils, release of neutrophilic enzymes, inhibition of the enzymes themselves, the generation of Hageman factor activity and members of the three systems activated by this component, and finally, inhibition of platelet and monocytic functions. These control assays are routinely performed in this laboratory.

8. Details of Experimental Design and Procedures: (Continued) - 5

c. The role of fibrin formation, and indirectly of its split products in the generation of pulmonary and glomerular lesions will be examined by depleting rabbits and rats of fibrinogen prior to injection of $^{125}I$ antibody. Two fibrinogenolytic enzymes, Venacil and Reptilase, derived from snake venoms will be employed, each of which exhibits marked specificity for fibrinogen. In our experiments, these enzymes can be injected safely 10 - 24 hours before an experiment resulting in marked depletion of fibrinogen but without measurable effect on the various systems noted under part b in the previous paragraph. Fibrinogen levels will be followed by use of thrombin-induced clotting and immunodiffusion assays.

d. The mechanism of neutrophil independent injury of pulmonary and glomerular membranes will also be studied by inhibition with the polypeptides Trasylol and pepstatin, and the inhibitors of trypsin derived from lima beans, soybeans and ovomucoid. These inhibitors are not specific to individual enzymes, but considerable information is being derived in this and other laboratories as to the enzymes in various mediation systems that are inhibited. This information is now being completed in the complement, fibrinolytic, kinin-forming and intrinsic clotting systems. A portion of these studies have been published (Appendix A). Their action on various leukocyte-derived enzymes is now being assessed. Each can be infused into experimental animals and the inhibitory effect on inflammatory lesions evaluated. In addition, several new enzyme inhibitors are available for evaluation, each of which inhibits kallikrein.

2. Studies of inflammatory injury mediated by leukocytes and other cells.

a. Neutrophils

We have recently devised a method of isolating and purifying peripheral neutrophils and infusing them into animals depleted of neutrophils. If the animals are prepared with antibody or immune complexes deposited along vascular membranes, the infused neutrophils reconstitute the reaction. We are now in a position to assess various functions of neutrophils in the development of inflammatory injury. The isolated neutrophils will be treated so as to block various functions thought to be important in their injurious capacity. They will then be infused into neutrophil depleted rabbits prepared with $^{125}I$ antibody which is bound to pulmonary or glomerular membranes. Injury will then be assessed as noted above. Comparison of the effect of inhibited neutrophils will be made with that of normal neutrophils and with animals in which neutrophil reconstitution is not made.

Several functions of neutrophils will be tested in this manner:

1) Immune adherence will be blocked by exposure of the neutrophils to freshly activated C3. The C3b will bind to the immune adherence reactive site on the neutrophil as determined by isotope marker on the C3b. The cells will be tested for their adherence capacity in vitro and for their ability to bind in test rabbits to pulmonary vascular membranes to which antibody and complement components are fixed. Adherence will be determined histologically.

8. Details of Experimental Design and Procedures: (Continued) - 6

As a corollary to the above, the time of decay of membrane-fixed C3b will be assessed by infusing neutrophils at various times into neutrophil-depleted rabbits, injected with anti-lung membrane or anti-GBM antibody. C3b, which binds rapidly after injection of antibody, is degraded by an inhibitory enzyme in plasma, the so-called conglutinin-activating factor (KAF). In preliminary tests, when neutrophils were replaced 24 hours after the injection of antibody, the cells did not adhere to the antibody and complement along vascular membranes. This indicated the complement had been altered during this time so as not to bind neutrophils. As control, cutaneous Arthus reactions will be applied at various times to insure that neutrophils are capable of binding to fresh antigen-antibody-complement sites.

These results will determine for the first time the decay rate of bound and activated complement components involved in the accumulation of neutrophils in vivo.

2) Inhibition of release of lysosomal constituents will be accomplished by treating the isolated neutrophils with $PGE_1$, methyl xanthines or iodoacetate. Each of these has been found in this and other laboratories to inhibit the release of lysosomes to the exterior upon stimulation of the cell with antigen-antbody complexes (Appendix A). The effect of these inhibitors will be confined to the neutrophils since the animal will not receive the inhibitor. Side effects will thereby be averted, and specificity of inhibition will be maintained.

These studies are of great importance in view of the role of neutrophils in tissue injury. And since α-1-antitrypsin is a strong inhibitor of neutrophilic protease (elastase) in man, these enzymes are strong candidates for the injury and resulting fibrosis of lung in α-1-antitrypsin deficiency states. In this regard, the studies noted in part 1d above on the inhibition of inflammation with pepstatin are of importance. We have recently observed that pepstatin inhibits human neutrophilic proteases.

b. Macrophages, mast cells and platelets. Studies have been conducted in this laboratory over the past three years on the involvement of alveolar macrophages, mast cells, basophils and platelets in tissue injury, and the mechanisms of release of vasoactive amines and other constituents from these cells (Appendix A). The biochemical basis for this release process will be further studied including an examination of the role of cyclic AMP (here apparently an important modulator of release), serine esterases and microtubules and microfilaments.

These studies will be performed with the aid of various inhibitors of microtubular formation (colchicine and vinblastine), microfilaments (cytochalasin B) and factors that affect levels of cAMP (β and α activating and inhibiting agents, methyl xanthines). The inhibitors will be applied to cells stimulated with aggregated immunoglobulins, and active moieties of the complement system. The techniques involved are in common use in this laboratory.

8. Details of Experimental Design and Procedures: (Continued) - 7

The platelet activating factor (PAF) (Appendix A) which we have found to be released from basophils will be further analyzed. PAF has been shown now to cause clumping of platelets with release of their vasoactive constituents and to induce an increase in vascular permeability. Recent preliminary studies have revealed that PAF is released from isolated, sensitized rabbit lung upon addition of antigen or anti rabbit IgE. This represents a new mediator of inflammation in lung the role of which must be determined. We now plan to study the mechanisms of its release from lung, immunoglobulin classes so involved and the means by which control of the release may be excercized. The cell of origin in the lung will be sought by desensitizing mast cells with anti IgE before adding antigen and vice-versa.

c. Interrelation between these systems. In the inflammatory reaction many of the humoral and cellular mediating processes are interrelated. Histamine can inhibit release from other cells and will be studied for its inhibition of neutrophil and platelet release. This could exert a controlling influence in vivo. Neutrophil and platelet accumulation is often accompanied by fibrin deposition as is seen in pulmonary lesions. Study of the connection between neutrophil release and initiation of coagulation will be undertaken. The affect of neutrophil enzymes and constituents on Hageman factor will be investigated along with the alternative possibility that platelets and neutrophils can be activated by components of the coagulation and kinin systems, just as they are by components of the complement system.

Summary of the relationship of these studies to pulmonary disease.

These studies will provide considerable understanding of the mechanisms of injury of the lung.

1. The importance of the intrinsic clotting system, platelets and neutrophils in the deposition of fibrin and injury of structure in acute pneumonitis will be assessed.

2. The increased vascular permeability in lung mediated by the kinin forming system and fibrin split products will be assessed.

3. The potential role of the platelet activating factor (PAF) in the development of pulmonary injury will be evaluated.

4. The mechanisms of release of injurious constituents of cells commonly found in pulmonary inflammation will be analyzed. Control mechanisms of the release process and inhibitors of the released injurious constituents will be examined.

Appendix A

Report of Progress 1970-73

1) The kinin forming and intrinsic clotting systems.

a) Purification and characterization.

In the past three years, in this laboratory, each component of the kinin forming system has been isolated and purified in both human and rabbit plasma. In addition, Factor XI of the intrinsic clotting system has been purified and characterized. This has been accomplished by a combination of precipitation in ammonium sulfate, anion exchange chromatography, gel filtration and block electrophoresis (1-7). In each case, purity has been determined by the appearance of single protein bands in polyacrylamide electrophoresis. Of greatest importance, the molecules have been obtained in inactive, precursor form. This has allowed studies to be performed on the mechanisms of activation of each component as will be noted below. The information obtained in these and future studies will form the basis of an eventual understanding of the role of the kinin forming and intrinsic clotting systems in disease. The point must be underscored that unequivocall evidence of the participation of the kinin forming and intrinsic clotting systems in health and disease are lacking. The reason behind this void of information lies in the unavailability until now of purified precursor components of these systems.

The following figure and table illustrate several characteristics of the precursor components and their interaction as determined in this laboratory:

Figure 1

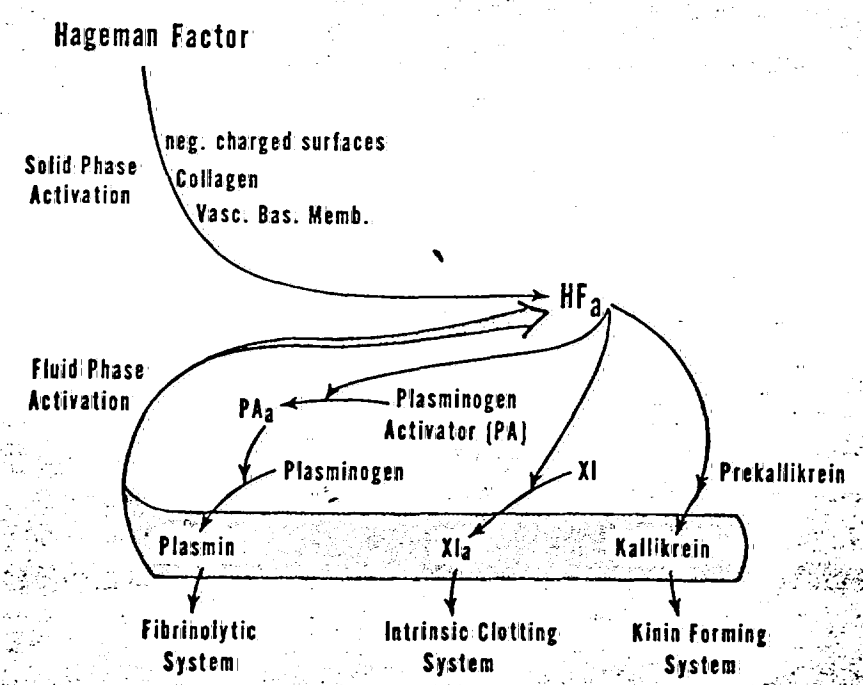

Table 1

Physical Properties of the Components of the Hageman Factor Activated Systems in Human and Rabbit Plasma

| | Hageman factor | Activated Hageman Factor (prekallikrein activator PKA) | Prekallikrein | Kininogen | Clotting Factor XI PTA | Plasminogen activator | Plasminogen |
|---|---|---|---|---|---|---|---|
| Human | | | | | | | |
| Molecular Wt. | 90,000 | 30,000 | 107,000 | 70,000 | 160,000 | 100,000 | 80,000 |
| Sed. Rate | 4.5 | 2.6 | 5.1 | 3.8 | 7 | | |
| Electroph. mobility | beta | pre-albumin | gamma | alpha | gamma | gamma | beta |
| Isoelectric pt. | 6.1 | 4.6 | | | >7.5 | 8.7-9.0 | 6.3-8.6 |
| Rabbit | | | | | | | |
| Molecular Wt. | 90,000 | 30,000 | 99,000 | 79,000 | | | |
| Sed. Rate | 4.5 | 2.6 | 4.5 | 3.8 | | | |
| Electroph. mobility | beta | pre-albumin | gamma | alpha | | | |
| Isoelectric pt. | 6.1 | 4.6 | 5.9 | 5.2 | | | |

b) Activation of individual components.

1. Hageman factor. Studies of the activation of Hageman factor have been aided by the ability to label the molecule with $^{125}I$. This has allowed us to determine that molecular cleavage of the parent molecule has taken place. A variety of proteolytic enzymes have now been found to activate Hageman factor: trypsin, kallikrein and plasmin. The parent molecule upon activation by trypsin cleaves into three subunits of identical size, 30,000 daltons (8, 9). The daughter molecules possess the capacity to activate purified prekallikrein and clotting factor XI and have been termed prekallikrein activator (PKA). In addition, upon reduction with 0.02 M dithiothreitol, $^{125}I$ labelled Hageman factor falls into three subunits of equal charge and size 30,000 daltons. This indicates that Hageman factor consists of three equal polypeptide chains held together by disulfide bonds at the far end of the polypeptide chains (since trypsin cleaves the parent molecule similarly).

Among the non-enzymatic activators, collagen and glomerular basement membrane have been found to induce activation of Hageman factor. This has been revealed by binding of $^{125}I$ Hageman factor to these insoluble membranes and by its subsequent activation of purified prekallikrein or clotting factor XI. By contrast, human immunoglobulin of each class and subclass upon aggregation fail to activate Hageman factor. Antigen-antibody complexes prepared in many different ways also failed to activate as well as rheumatoid factors after combination with aggregated $\gamma$ globulin. We have thus gained strong evidence against immunologic activation of this important factor.

Enzymatic activation of Hageman factor has been established. Kallikrein was found to activate Hageman factor in fluid phase with great sensitivity. Plasmin and Factor XI were found to activate but required 5 and 10 fold greater amounts respectively. The fluid phase activation gained importance when it was found that less than 1% of available Hageman factor binds to and is activated by the potent surface activator kaolin in the presence of undiluted plasma. Kallikrein has been found to promote clotting of plasma (10), in part, at least, through the activation of Hageman factor. The importance of kallikrein in activation of Hageman factor and the three systems was determined by studies of plasma exhibiting the Fletcher Factor deficiency. When exposed to glass, this plasma fails to form kinin, plasmin and clots with a greatly increased time. We determined that the missing factor was actually prekallikrein by functional and immunochemical means (11). Addition of prekallikrein restored the clotting, plasmin and kinin forming capacities.

PKA possesses enzymatic activity, being capable of splitting substituted esters of lysine (AGLMe, TLMe, CBZLMe, ALMe) but not esters of arginine (BAEe, BAMe, BAPN, AAMe). Analysis of the initial rate of activation of prekallikrein by PKA supports the enzymatic nature of the reaction. In addition, lima bean trypsin inhibitor, DFP and phenyl-methyl-sulfonyl-fluoride block the activity of PKA.

Antibody has been prepared to human Hageman factor, and quantitative immune diffusion analyses conducted. Initial studies reveal that normal human plasma contains 165 µg Hageman factor/ml. Fluorescein labeling of the antibody has been successfully performed. We are now in a position to analyze quantitatively for the first time the levels of Hageman factor in plasma and fluids in various disease states, turnover times of the $^{125}I$ labelled molecule and the binding of Hageman factor at sites of injury.

2. Prekallikrein. PKA cleaves rabbit prekallikrein into two fragments of 86,000 and 11,000 daltons (6). The larger of the two possesses kallikrein activities (arginine esterase and kinin release from kininogen). Human kallikrein is also cleaved, but reduction of the molecule is required to demonstrate that cleavage has occurred.

3. Factor XI of the intrinsic clotting system in human plasma has been isolated and purified. This molecule of 160,000 daltons consists of two polypeptide chains of equal size joined by disulfide bonds. Antibody to Factor XI has been prepared. Upon activation by PKA, Factor XI hydrolyzes synthetic amino acid substrates of arginine and lysine and is inhibited by DFP (12).

2) Neutrophil-mediated immunologic injury.

One of the in vivo models which will be employed in this study is the experimental arthritis which has been developed in this laboratory (13). This comprises a reversed passive Arthus reaction which is initiated in the rabbit stifle joint. The injury can be quantitated and requires complement and neutrophils. Most importantly, by replacing neutrophils into the joint of a neutrophil-depleted rabbit, the injury has been restored (13, 14). This reaction requires the migration of the neutrophils from joint space to the site of immune complex formation in the blood vessel walls. The migration was shown to be dependent upon C3 and C6 and is the first clear indication in vivo of the role of complement-derived chemotactic factors. Once the neutrophils have reached the complexes, phagocytosis occurs and it is presumed that tissue injury again follows the release of lysosomal constituents from the neutrophils.

More recently, experiments have indicated that it is also possible to re-establish lesions of nephrotoxic nephritis in neutrophil-depleted rabbits by intravenous injections of neutrophils. The neutrophils became bound in glomeruli only when rabbits were pretreated with nephrotoxic antibody (selected to produce injury in the presence of neutrophils). The neutrophils became bound in their first pass through the glomeruli. Injury to the glomerular basement membrane and proteinuria rapidly ensued. In other studies, three different types of antibody were obtained from fractions of sheep anti-rabbit glomerular basement membrane antiserum. One of these induced the neutrophil-dependent glomerulonephritis which will be used in these replacement studies, another produced a neutrophil-independent injury the pathogenesis of which is currently under investigation, and the third became bound to the basement membrane but induced no damage at all (15, 16).

Release of granule enzymes from neutrophils phagocytosing immune complexes and particles has been demonstrated by a number of workers. The mechanism of release in vitro is being studied in this laboratory and has already been shown to follow degranulation into a phagocytic vacuole which may be open, or may later become open, to the exterior (17, 18, 19).

In addition, study of neutrophils in vitro (17, 18, 19) has revealed that granule enzymes of a potentially injurious nature are released from the cells when they are adherent to antibody or complement bound to non-phagocytosable surfaces (like the basement membranes). The in vitro observation showed that in the system employed, direct exocytosis of the neutrophil granules was the mechanism of release. It now becomes important to demonstrate this in vivo. Studies of these reactions in vitro are continuing (16, 20) and are beginning to define the biochemical reactions of the cells which culminate in exocytosis of granules ("degranulation").

The release is inhibited by agents which increase intracellular cyclic AMP (prostaglandins, phosphodiesterase inhibitors), which inhibit glycolysis, or by diisopropyl fluorophosphate which inhibits serine esterases. Calcium is also required for the release. These phenomena with neutrophils closely resemble release mechanisms of other cells.

Continuing studies on the mechanisms of deposition of circulating immune complexes have revealed that the increased vascular permeability, which is essential for deposition of the circulating complexes in vessel walls, may result from the interaction of sensitized leukocytes with antigen and platelets. In the reaction, the platelets clump and release vasoactive amines (20, 22). The leukocyte involved has now been shown to be a basophil and the reaction may be transferred to leukocytes of a normal rabbit with IgE antibody (22, 23, 24). Using cells from human beings sensitive to various antigens, data are emerging that indicate a similar reaction system (25). A soluble factor (PAF) was found to be released from the sensitized basophils and this mediated the clumping of platelets and release of their vasoactive amines (24, 26). This factor has been shown to bind readily to albumin, and to be extractible with ethyl alcohol (22, 25).

## References


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